学位論文

Macrophage/microglia-derived IL-1β induces glioblastoma growth via the STAT3/NF-κB pathway
（マクロファージ由来の IL-1β は STAT3/NF-κB 経路を介して膠芽腫の増殖を誘導する）

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Abstract

Glioblastoma is a glioma characterized by highly malignant features. Numerous studies conducted on the relationship between glioblastoma and the microenvironment have indicated the significance of tumor-associated macrophages/microglia (TAMs) in glioblastoma progression. Since interleukin (IL)-1β secreted by TAMs has been suggested to promote glioblastoma growth, we attempted to elucidate the detailed mechanisms of IL-1β in glioblastoma growth in this study. A phospho-receptor tyrosine kinase array and RNA sequencing studies indicated that IL-1β induced the activation of signal transducer and activator of transcription-3 and nuclear factor-kappa B signaling. Glioblastoma cells stimulated by IL-1β induced the production of IL-6 and CXCL8, which synergistically promoted glioblastoma growth via signal transducer and activator of transcription-3 and nuclear factor-kappa B signaling. By immunohistochemistry, IL-1β expression was seen on TAMs, especially in perinecrotic areas. These results suggest that IL-1β might be a useful target molecule for anti-glioblastoma therapy.

Keywords: Glioblastoma, macrophage, IL-1β, IL-6, CXCL8

Introduction

Glioma is the most common tumor of the primary central nerve system in human [1]. Among glioma, glioblastoma (GBM) is the most frequent, and it is characterized by highly malignant features. GBM shows rapid growth and infiltration; thus, standard treatment, including maximum resection of the tumor mass and chemo-radiotherapy (temozolomide and radiation), is performed as standard therapy [2-4]. However, the mean 5-year survival rate is still less than 10%. Thus, further advancements are needed to improve patient prognosis.

In recent years, the tumor microenvironment (TME) has been receiving a lot of attention. Macrophages/microglia are the main component of immune cells that make up the TME [5]. Resident microglia are the only leukocytes in the brain parenchyma during the steady state; however, monocyte-derived tumor-associated macrophages (TAMs) replace the microglia as the GBM volume increases [6]. TAMs in the TME

promote cancer cell proliferation, immunosuppression and angiogenesis, leading to GBM growth [7,8]. A high density of TAMs with the M2-like phenotype was found to be associated with GBM proliferation and a worse clinical course in a human study [9]. TAMs have become increasingly recognized as a cancer therapy target, e.g., in combination therapy with an immune checkpoint inhibitor and chemo-radiotherapy [10-12].

GBM-derived factors, including periostin, are known to recruit TAMs to the TME and induce the M2-like phenotype in TAMs, which promote GBM growth and invasion [13]. We previously demonstrated that TAMs promoted GBM growth by secreting soluble factors, such as heparin-binding epidermal growth factor (EGF)-like growth factor and interleukin (IL)-1β [14]. Heparin-binding EGF is a well-known molecule that activates tumor cells via the epidermal growth factor receptor / Hypoxia inducible factor alfa (EGFR/HIF-1α) pathway [15,16]. IL-1β, one of the isoforms of the IL-1 family, is a pro-inflammatory cytokine that is secreted by myeloid cells. Sasaki et al. previously demonstrated that the gene expression level of IL-1β was increased in grade IV glioma as compared to grade II and III glioma, and IL-1β protein expression was detected in both tumor cells and TAMs [17]. A recent study showed that IL-1β expression was up-regulated in GBM stem cells, and a high gene expression signature for IL-1β was associated with a worse clinical course in GBM [18]. However, the detailed mechanisms of IL-1β-induced GBM growth remains unclear. Therefore, we examined the involvement of IL-1β in GBM growth by means of *in vitro* studies.

Materials and Methods

Cell cultures

The human GBM cell lines T98G and U251 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). All cells were cultured in DMEM/F12 (Wako, Tokyo, Japan) with 10% fetal bovine serum (Invitrogen, Waltham, MA). Three-dimensional (3D) cell culture was performed as described previously [19,20]. In brief, cell lines (100 cells/100 μL/well) were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum and SphereMax (Wako) using a 96-well ultra-low attachment plate (Corning, Corning, NY, USA) for 10 days. Total cell viability was evaluated by analyzing

the cellular ATP activity using a Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA). Recombinant CXCL8, IL-6 and IL-1β were obtained from Wako. Inhibitors for STAT3 (WP1066, Santa Cruz, Dallas, TX) and NF-κB (BAY11-7082, WAKO) were also used.

RNA sequencing (RNA-seq) transcriptome analysis

Cell lines were treated with IL-1β (0.1 ng/mL) for 24 h, and total RNA was isolated with RNAiso Plus (Takara Bio, Shiga, Japan). For RNA-seq of the GBM samples and the GBM with IL-1β samples, total RNA was prepared by Trizol extraction (Thermo Fisher, Waltham, MA, USA), and the quality of total RNA was confirmed by BioAnalyzer 2100 (RIN >9). DNA libraries were prepared according to the Illumina TruSeq protocol using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), and sequenced by Illumina NextSeq 500 (Illumina) using the NextSeq 500/550 High Output v2 Kit (Illumina) to obtain single-end 75-nt reads. RNA-Seq Data Analysis Resulting reads were aligned to the human genome (UCSC hg19) using STAR ver.2.6.0a after trimming to remove adapter sequences and low-quality ends using Trim Galore! v0.5.0 (cutadapt v1.16). Gene expression levels were measured as transcripts per million as determined with RSEM v1.3.1. Gene Ontology enrichment analysis and pathway enrichment analysis were performed using multiple databases: PANTHER (http://www.pantherdb.org/), DAVID (https://david.ncifcrf.gov/), and Metascape (http://metascape.org/gp/index.html#/main/step1).

Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from cells using RNAiso Plus (Takara Bio). RNA was reverse-transcribed by means of a PrimeScript RT Reagent Kit (Takara Bio). PCR amplification of the resulting cDNA was carried out with Hot Start Version (Takara Bio) using a hot start of 98°C for 10 s, 55°C for 15 s, and 68°C for 30 s, followed by 30 PCR cycles using iCycler (Bio-Rad, Hercules, CA, USA).

Phospho-receptor tyrosine kinase (RTK) array

Phospho-RTK array analysis was performed using the Human Phospho-RTK Array Kit

(ARY 001, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Enzyme linked immunosorbent assay (ELISA)

The concentration of IL-1β in medium was tested by ELISA kit (BioLegend, San Diego, CA). Before collecting the supernatant medium from cell culture, cells were treated by ATP as described previously (21).

RNA in situ hybridization (ISH)

RNAScope Duplex kit (Advanced Cell Diagnostics, Newark, CA) was used for detecting the mRNA expression on paraffin sections.

Immunohistochemistry (IHC)

Paraffin-embedded tissue samples were prepared from GBM patients diagnosed at Kumamoto University Hospital, Japan. Paraffin sections were subjected to IHC using anti-Iba1 antibody (mouse monoclonal, Wako) and anti-IL-1β antibody (rabbit polyclonal, Bs-0812R, Bioss Antibodies, Woburn, MA, USA) as the primary antibodies. Alexa 488-labeled anti-mouse Ig and Alexa 546-labeled anti-rabbit Ig (Invitrogen, ThermoFisher) were used as the secondary antibodies.

Western blot

Cells were solubilized in Tris buffer containing 2% sodium dodecyl sulfate and 10% glycerol. The amount of protein was quantified using the bicinchoninic acid assay. Equal amounts of protein were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and subsequently transferred to a polyvinylidene fluoride membrane. The following mouse antibodies were used for western blotting: anti-pSTAT3 (Y705, Cell Signaling, Danvers, MA, USA) and anti-pNF-κB (S536, Cell Signaling). The membranes were re-blotted with an anti-β-actin antibody as an internal control.

Statistical analysis

Statistical analysis was carried out using JMP7 software (SAS Institute, Chicago, IL,

USA) and StatMateIII (ATOMS, Tokyo, Japan). For all analyses, $p < 0.05$ was considered to be statistically significant.

Results

IL-1β induced GBM cell growth in the 3D cell culture system.

Since we previously showed that IL-1β induced the growth of T98G GBM cells in a 3D culture system [14], we tested whether a similar protumor effect of IL-1β would be observed in U251 GBM cells. As shown in Figure 1A and 1B, IL-1β significantly induced the cell growth of U251 cells in 3D culture as effectively as it did T98G cells.

*Signal transducer and activator of transcription-3 (S*TAT3) *and NF-κB signaling may have activated IL-1β.*

Next, signaling molecules activated by IL-1β stimulation were examined using the RTK array kit. The assays using T98G and U251 cells indicated that STAT3 and PYK2 were strongly activated by IL-1β (Figure 2A). The PYK2 signaling pathway has been shown to be associated with the invasion and migration of GBM cells [22]; however, there has been no report describing a correlation between the PYK2 signaling pathway and glioma cell growth. Since it is well-known that the STAT3 pathway is associated with tumor growth and tumorigenesis in GBM [23], STAT3 signal was investigated in the present study.

Next, the effect of IL-1β on gene expression was examined by RNA-seq (Figure 2B). The up-regulated genes and down-regulated genes in T98G and U251 cells are listed in Table 1. The results suggested that IL-1β activated NF-κB-related signaling (Figure 2C).

IL-6 and CXCL8 synergistically accelerated GBM growth.

Among the listed genes in Table 1, IL-6 and CXCL8 are well-known molecules involved in tumorigenesis of malignant tumor including GBM [24,25]. IL-6 is closely linked to STAT3 signal activation [26], whereas, CXCL8 activated both STAT3 and NF-κB-related signals [27]. Based on these findings, we selected IL-6 and CXCL8 as potential growth factors for GBM cell lines. The result of RNA-seq was confirmed by real-time PCR. IL-

6 and CXCL8 were significantly up-regulated by IL-1β in T98G and U251 cells (Figure 3A). The genes for IL-1β and CCL2 were also listed as up-regulated genes in Table 1, and the overexpression of these genes in T98G and U251 cells was confirmed by real-time PCR (Figure 3A). Then, we tested whether IL-6 and CXCL8 would affect GBM cell growth in 3D culture. Neither IL-6 nor CXCL8 induced tumor cell growth; however, co-stimulation by both cytokines significantly promoted tumor cell growth (Figure 3B).

STAT3 and NF-κB signaling was involved in GBM growth.

As shown in Figure 2, STAT3 and NF-κB signaling was suggested to be critical for IL-1β-induced GBM cell growth, so we tested whether inhibitors of these signals would be effective in inhibiting GBM cell growth. We found that the IL-1β-induced tumor cell growth was significantly abrogated by the STAT3 inhibitor (STAT3 inhibitor, WP1066) and NF-κB inhibitor (BAY11-7082; Figure 4A). Western blot analysis showed that IL-1β activated both STAT3 and NF-κB signaling (Figure 4B) in both T98G and U251 cells. NF-κB signaling was activated by 3 h of stimulation with IL-6 and CXCL8. In contrast, the activation of STAT3 signaling differed between the T98G and U251 cells (Figure 4B); in U251 cells, STAT3 signaling was activated after 24 h of stimulation with IL-6 and CXCL8, but in T98G cells, STAT3 signaling was activated by IL-6, but not by CXCL8.

Increased IL-1β expression was observed in macrophages co-cultured with GBM cells

Cell culture study in figure 3A indicated that IL-1β was up-regulated in stimulated GBM cells. Then, we tested which cells are main source of IL-1β in GBM microenvironment. GBM cells and macrophages were stimulated with CM of macrophages and GBM cells respectively to investigate the indirect influences between GBM cells and macrophages. In addition, GBM cells and macrophages were directly co-cultured to test the significance of direct cell-cell contact between GBM cells and macrophages (Figure 5A). As the result of ELISA assay, CM-stimulation induced slight up-regulation of IL-1β in both cells and notably significant increased IL-1β production was observed in direct co-culture (Figure 5B). ISH assay using cell block specimens of co-cultured cells indicated Iba1-positive macrophages express significant higher level of IL-1β more than GBM cells (Figure 5C). IL-1β in GBM cells were not influenced by CM of macrophages, whereas, IL-1β in

macrophages was increased by CM of GBM cells (Figure 5D). IL-1β expression was higher in activated macrophages than GBM cells, although IL-1β expression was detected both in macrophages and GBM cells (Figure 5C and 5D).

IL-1β expression was observed in TAMs infiltrating perinecrotic areas.

An analysis using open-source databases was performed to investigate the significance of IL-1β expression in GBM. The Brain Tumor Immune Micro Environment (TIME) database (https://joycelab.shinyapps.io/braintime/) [28] showed that higher expression of IL-1β was observed in neutrophils and TAMs than in lymphoid cells and tumor cells (Figure 5A). There was no significant difference in IL-1β expression between microglial cells and monocyte-derived cells (Figure 6A). The Human Protein Atlas database (https://www.proteinatlas.org/) showed that IL-1R expression, but not IL-1β expression, in GBM was associated with a worse clinical course (Figure 6B). There were significant correlations between IL-1β and macrophage markers (Figure 6C). The Ivy Glioblastoma Atlas (https://glioblastoma.alleninstitute.org/) showed that strong IL-1β expression was observed in perinecrotic areas (Figure 6D). The distribution of IL-1β was tested in 10 cases of human GBM with necrotic area and viable tumor area by double-IHC and dual-colored ISH. Higher expression of IL-1β protein and mRNA were detected in TAMs than those in GBM cells, and high IL-1β protein and mRNA were preferentially detected in perinecrotic areas in 6 cases (Figure 7A-C).

Discussion

In the present study, we demonstrated the detailed mechanisms of IL-1β-induced tumor cell proliferation and the production of IL-1β by TAMS in the TME. Various factors derived from TAMs have been shown to induce GBM cells, as described in the introduction, but few studies have reported on the significance of IL-1β in GBM proliferation. In the present study, GBM proliferation was not changed by IL-1β in the 2D culture study, but it was significantly promoted by IL-1β in T98G and U251 cells. The results from T98G cells were consistent with those of previous reports; however, this is the first time that the protumor effect of IL-1β on U251 cells in 3D culture has

been reported.

The mechanisms underlying the protumor effect of IL-1β have been reported in solid tumors other than GBM. In pancreatic cancer, TAM-derived IL-1β was suggested to induce the downregulation of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which degrades prostaglandin [29]. The level of 15-PGDH is frequently decreased in several types of cancer [30,31]. In our unpublished data, 15-PGDH was not expressed in GBM cell lines and tissues. In the present study, STAT3 and NF-κB signaling was shown to be involved in IL-1β-induced GBM cell growth. A previous study has shown that IL-1R-mediated signaling contributed to NF-κB activation, which subsequently induced CXCL8 production [32]. However, in the present study, it was shown that CXCL8 directly activated NF-κB signaling in GBM cells. The synergistic effect of IL-1β and CXCL8 on the activation of NF-κB-related signaling might accelerate GBM growth.

The synergistic effect of IL-6 and CXCL8 on GBM growth in the 3D culture system was demonstrated in the present study, although neither IL-6 nor CXCL8 alone was associated with GBM growth. Strong STAT3 activation was induced by IL-6, whereas NF-κB activation was induced by CXCL8 in T98G cells; this suggested that IL-6 and CXCL8 synergistically activated the STAT3 and NF-κB pathway. In U251 cells, STAT3 and NF-κB were both activated by either IL-6 or CXCL8. Yet, unknown mechanisms might contribute to the synergistic effect of IL-6 and CXCL8 in GBM cells. The cell culture study in which the STAT3 inhibitor or NF-κB inhibitor abrogated the IL-1β-related GBM growth indicated that both STAT3 and NF-κB are necessary for the GBM growth-inducing effect of IL-1β.

The detailed distribution of microglial TAMs and monocyte-derived TAMs in GBM has not yet been clarified. However, Klemm et al. have shown that in GBM with a mutation in isocitrate dehydrogenase, microglial TAMs were more numerous than monocyte-derived TAMs, but the opposite was seen in GBM with wild-type isocitrate dehydrogenase [28]. Their gene expression data published on the Brain TIME database showed that IL-1β expression was higher in microglial TAMs, monocyte-derived TAMs, and neutrophils than in lymphoid cells and tumor cells. The gene expression data from the Protein Atlas showed a significant correlation between IL-1β and macrophage

markers, indicating that the gene expression of IL-1β was predominantly associated with TAM infiltration. Data from the Ivy Glioblastoma Atlas showed that IL-1β expression was higher in perinecrotic areas than in other areas. Taken together, IL-1β was suggested to be expressed on TAMs in perinecrotic areas, and the IHC results showed IL-1β expression on TAMs in perinecrotic areas. Damage-associated molecular patterns were suggested to induce IL-1β expression in TAMs via toll-like receptors [33].

In conclusion, TAM-derived IL-1β was associated with GBM cell growth via STAT3 and NF-κB activation, which was partially mediated by IL-6 and CXCL8 in an autocrine manner (Figure 8). TAM-derived IL-1β appeared to up-regulate IL-1β and CCL2 production in GBM cells, and CCL2 in turn accelerated TAM infiltration into the GBM microenvironment. IL-1β expression was predominantly detected in perinecrotic areas, and damage-associated molecular patterns from necrotic cells were suggested to induce IL-1β expression in TAMs. Since anti-IL-1β antibody has been tested and used in clinical trials, anti-IL-1β therapy may be feasible and useful as a therapy for GBM.

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Disclosure

None of the authors have any conflicts of interest in association with this manuscript.

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients provided informed consent in accordance with protocols of the Kumamoto University Review Board, and the study design was approved by the Kumamoto University Review Board (approval no. 1174).

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Figure legends:

Figure 1. Interleukin (IL)-1β and three-dimensional (3D) culture of glioblastoma (GBM) cell lines. T98G and U251 cells (200 cells/well) were cultured in 96-well plates for 10 days as described in the Materials and Methods. Colonies were identified (A), and total viable cell numbers were evaluated by an ATP assay (n = 4 to 6) (B). Analysis of variance was performed. *; $p < 0.05$.

Figure 2. Phospho-receptor tyrosine kinase (RTK) array and RNA-sequencing (RNA-seq) of GBM cell lines stimulated with IL-1β. The T98G and U251 cell lines were stimulated with IL-1β for 24 h in 2D culture. (A) Signal densities of the RTK array were quantified by Image J software, and relative signal densities are presented. (B) Up-regulated and down-regulated genes were identified by RNA-seq. The diagrams show the normalized gene expression data in transcripts per million (TPM) for 38 genes (>20 TPM, >2 folds) that were identified as IL-1β-induced genes. (C) Gene Ontology enrichment analysis was applied to each molecular complex detection (MCODE) network to assign “meanings” to the network component.

Figure 3. Cytokines up-regulated by IL-1β. (A) Four genes that were strongly up-regulated by IL-1β in the RNA-seq analysis were confirmed by real-time polymerase

chain reaction (n = 3). (B) 3D culture of GBM cells (T98G, U251) with or without IL-6/CXCL8 (1 ng/mL each), and the total cell numbers were evaluated by an ATP assay (n = 6). *; $p < 0.05$.

Figure 4. The significance of STAT3 and NF-κB signaling in IL-1β-induced GBM growth. (A) A STAT3 inhibitor (10 μM) or NF-κB inhibitor (5 μM) was added to the 3D culture system, and cell viability was tested by an ATP assay (n = 4 to 6). Since inhibitors were dissolved in dimethyl sulfoxide, the same amount of dimethyl sulfoxide was added for the control and IL-1β groups. *; $p < 0.05$. (B) GBM cells were treated with IL-6 (1 ng/mL), CXCL8 (1 ng/mL), and IL-1β (0.1 ng/mL), and the phosphorylation of STAT3 and NF-κB was evaluated by western blot analysis.

Figure 5. Increased IL-1β expression in macrophages. GBM cells and macrophages were cultured with or without cell-cell interaction (A), and IL-1β production in supernatant was tested by ELISA (n=4) (B), ISH (C), and real-time PCR (n=3) (D). Scale bar; 10μm. *; $p < 0.05$.

Figure 6. Potential IL-1β expression in TAMs. (A) Reference IL-1β expression levels in non-hematopoietic cells, including tumor cells, endothelial cells, tumor-associated macrophages/microglia (TAMs), neutrophils, and lymphocytes, were obtained from the Brain TIME database (https://joycelab.shinyapps.io/braintime/). Kaplan-Meyer analysis (B) and Spearman's correlation test (C) were performed with data from the Human Protein Atlas database (https://www.proteinatlas.org/). (D) Reference IL-1β expression levels for each histological component were obtained from the Ivy Glioblastoma Atlas (https://glioblastoma.alleninstitute.org/).

Figure 7. IL-1β expression in GBM tissues. (A) Representative IHC figures of Iba1 (a marker for microglia and macrophages) and IL-1β in human GBM specimens were presented. (B) Double-IHC of IL-1β and Iba1 was performed in GBM sections. Scale bar; 50μm. (C) Dual-ISH figures of IL-1β (Green) and Iba1(Red) were presented. Scale bar; 10μm.

Figure 8. Hypothetical scheme of the IL-1β-mediated interaction between TAMs and GBM. Damage-associated molecular patterns (DAMPs) stimulate TAMs, which secrete IL-1β. GBM cells activated by IL-1β secrete IL-1β, IL-6, CXCL8, CCL2, and other molecules. IL-1β, IL-6, and CXCL8 contribute to GBM growth via STAT3 and NF-κB activation. CCL2 accelerates TAM migration into GBM tissues.

Table 1; Up-regulated and down-regulated genes by IL-1β

Up-regulated by IL-1β	down-regulated by IL-1β
AKR1B1, C1QTNF1, CCL2, CD82, CSF2, CXCL3, CXCL8, IER3, IKBKE, IL11, IL1B, IL32, IL6, IL7R, IRAK2, KYNU, MAN1A1, MT1E, NFKB2, NFKBIA, NFKBIZ, OAF, P4HA2, PIK3CD, PLAUR, PTX3, RELB, S100A3, SERPINB8, SLC2A6, SLC39A14, SOD2, SRGN, TMEM132A, TNFAIP3, TNIP1, VEGFC, ZC3H12A	CFI, EFEMP1, NR2F1, PI15

Figure 1

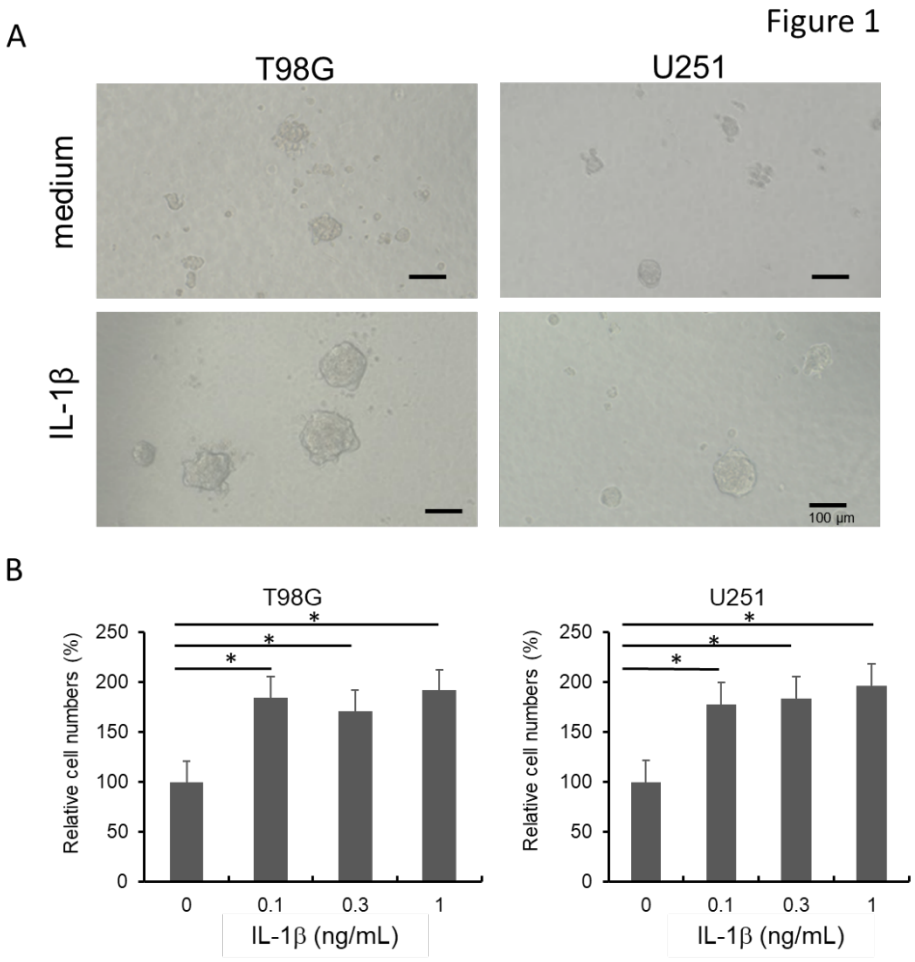
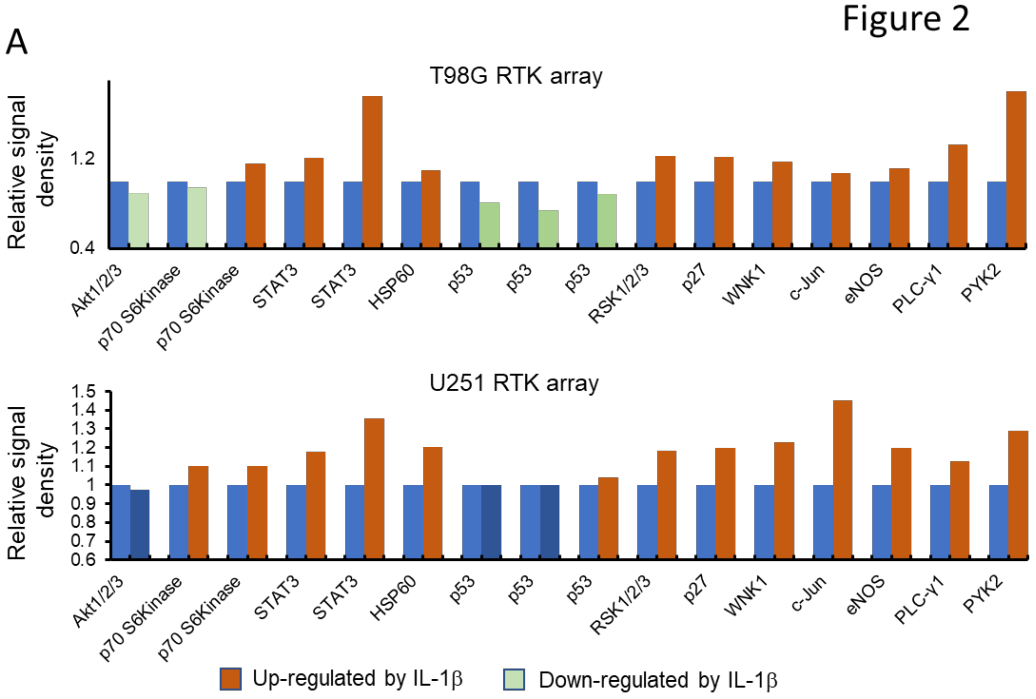
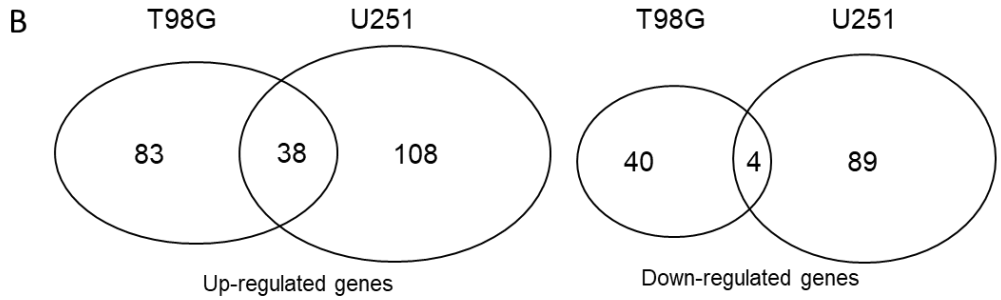


Figure 2





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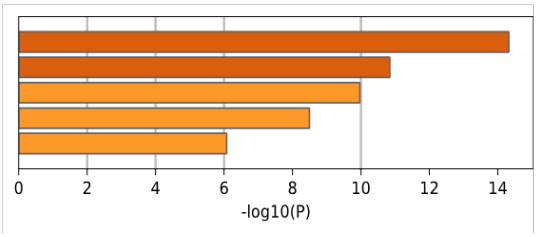


ko04567: IL-17 signaling pathway
hsa04060: Cytokine-cytokine receptor interaction
hsa04625: c-type lectin receptor signaling pathway
ko04064: NF-kappa B signaling pathway
hsa05200: Pathways in cancer

Figure 3

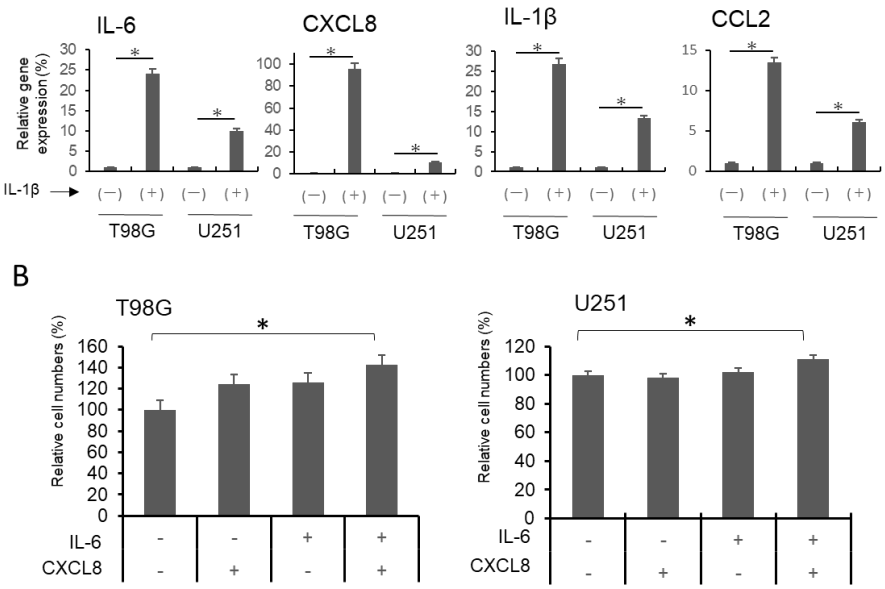
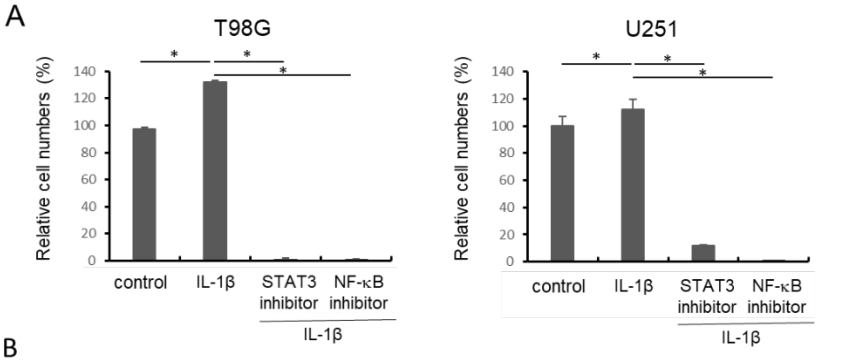


Figure 4



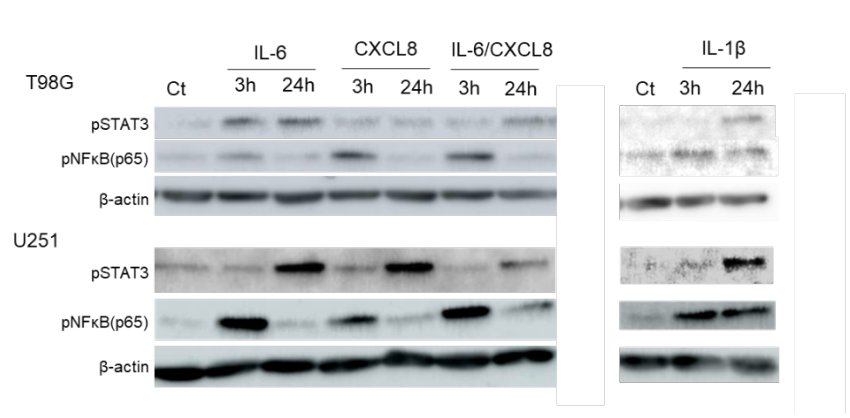


Figure 5

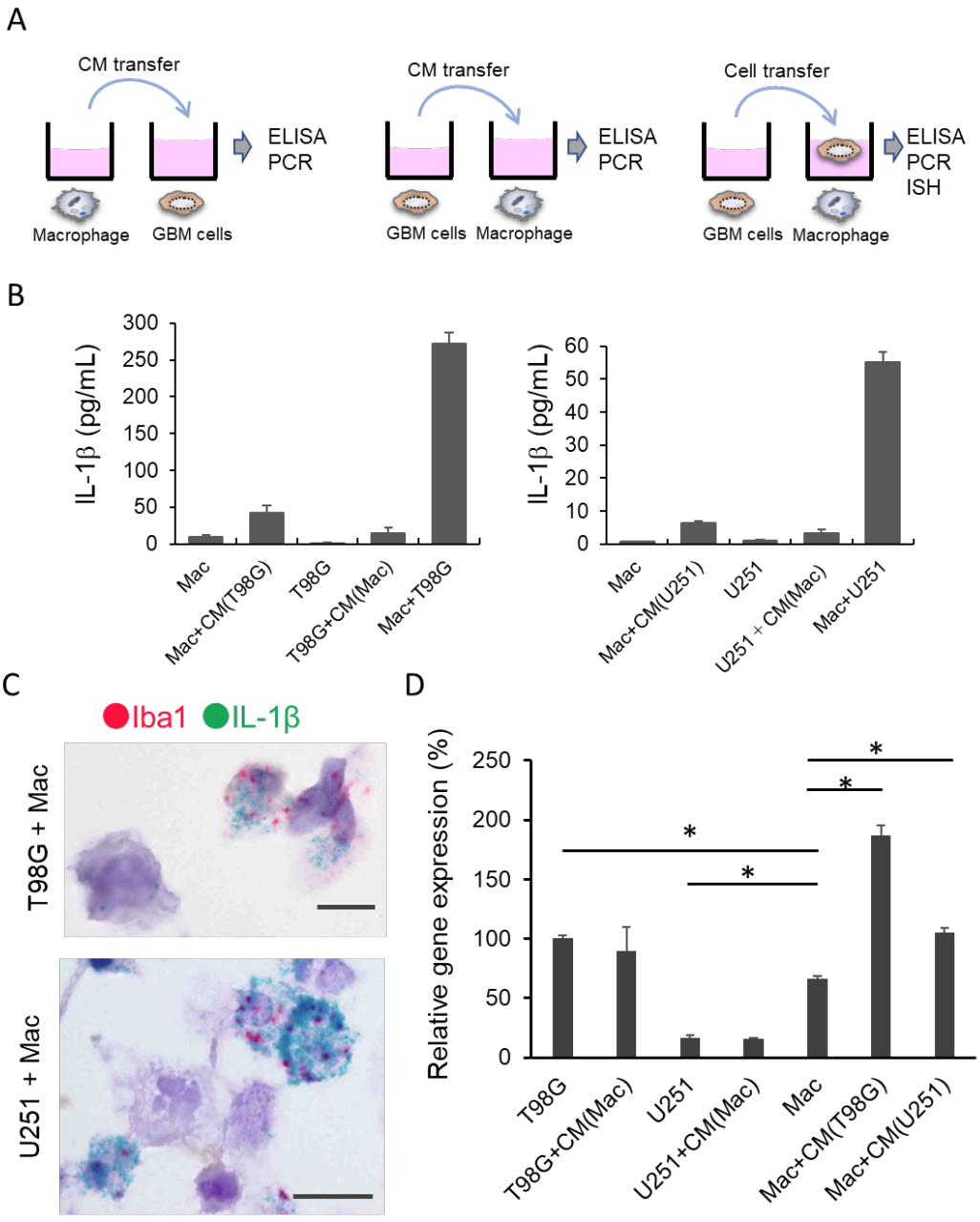

A
CM transfer
ELISA
PCR
Macrophage
GBM cells
CM transfer
ELISA
PCR
GBM cells
Macrophage
Cell transfer
ELISA
PCR
ISH
GBM cells
Macrophage
B
IL-1β (pg/mL)
300
250
200
150
100
50
0
Mac
Mac+CM(T98G)
T98G
T98G+CM(Mac)
Mac+T98G
IL-1β (pg/mL)
60
50
40
30
20
10
0
Mac
Mac+CM(U251)
U251
U251+CM(Mac)
Mac+U251
C
Iba1
IL-1β
T98G + Mac
U251 + Mac
D
Relative gene expression (%)
250
200
150
100
50
0
*
*
*
*
T98G
T98G+CM(Mac)
U251
U251+CM(Mac)
Mac
Mac+CM(T98G)
Mac+CM(U251)

Figure 6

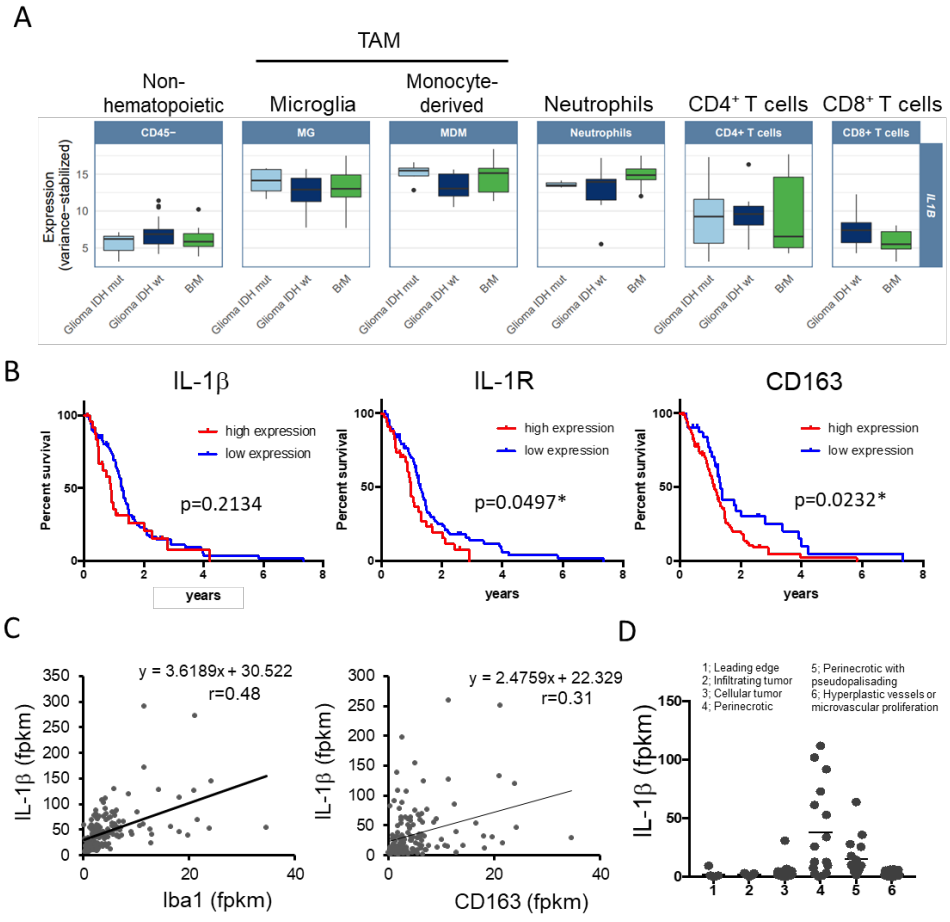

A
TAM
Non-hematopoietic
Microglia
Monocyte-derived
Neutrophils
CD4⁺ T cells
CD8⁺ T cells
CD45−
MG
MDM
Neutrophils
CD4+ T cells
CD8+ T cells
IL1B
Expression (variance-stabilized)
Glioma IDH mut
Glioma IDH wt
BrM
B
IL-1β
IL-1R
CD163
high expression
low expression
p=0.2134
p=0.0497*
p=0.0232*
Percent survival
years
C
y = 3.6189x + 30.522
r=0.48
y = 2.4759x + 22.329
r=0.31
IL-1β (fpkm)
Iba1 (fpkm)
CD163 (fpkm)
D
1; Leading edge
2; Infiltrating tumor
3; Cellular tumor
4; Perinecrotic
5; Perinecrotic with pseudopalisading
6; Hyperplastic vessels or microvascular proliferation

Figure 7

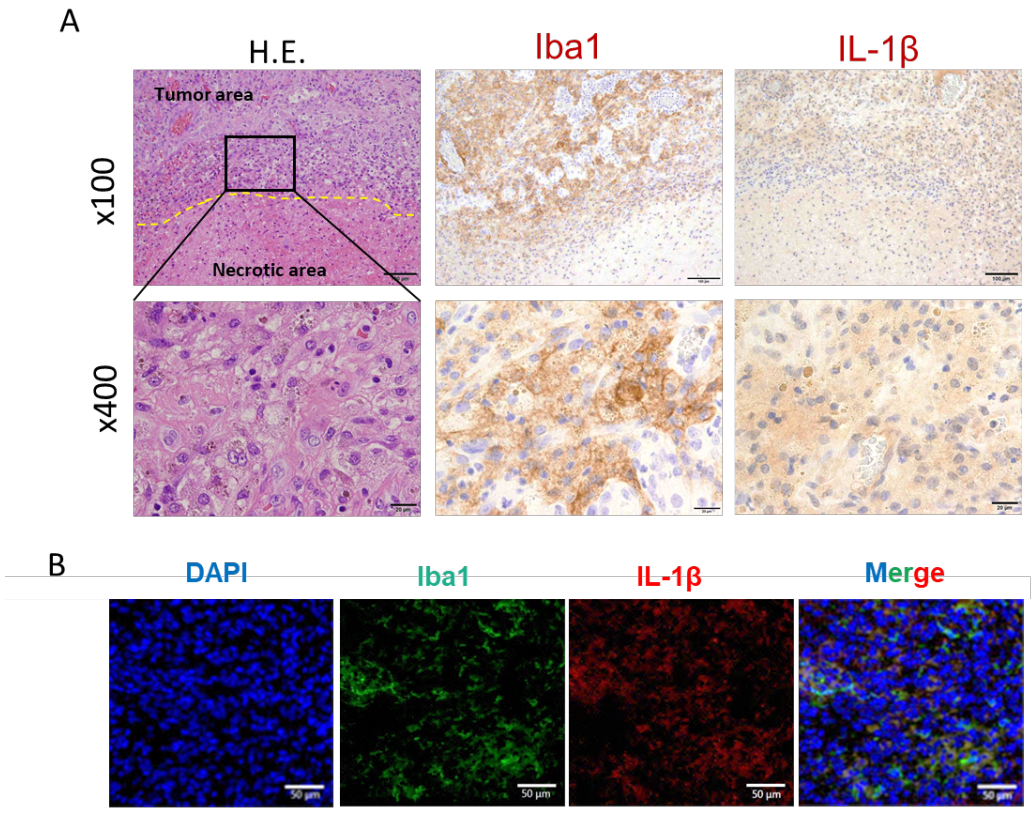


Figure 8